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Heterogeneity in E2 Region of GBV-C/Hepatitis G Virus and Hepatitis C Virus

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GB virus C/hepatitis G virus (GBV-C/HGV) is related distantly to hepatitis C virus (HCV). HCV has a hypervariable region (HVR), and exists as quasispecies in vivo. Although GBV-C/HGV also has replaceable amino acids in the presumed antigenic region, the existence and fluctuation of population of heterogeneous virus have not been evaluated. In this study, the heterogeneity of GBV-C/HGV and HCV was investigated by the single-strand conformation polymorphism (SSCP) analysis in six concomitantly infected patients. Two patients were observed for 4 years without any treatment, and four were treated with interferon-α (IFN). By SSCP analysis, amplicons of GBV-C/HGV RNA were separated into 1–5 bands on gels for each patient. The amplicons had different nucleotide but the same amino acid sequences in the presumed antigenic region. The amplicons of HCV RNA, separated into 1–4 bands, had different nucleotide and amino acid sequences in the HVR. In the two patients without treatment, the predominant strain of GBV-C/HGV was unchanged for the 4 years. In the four patients administered IFN, some strains of GBV-C/HGV disappeared after IFN therapy, whereas other strains persisted. The mean genetic distance among GBV-C/HGV strains represented by SSCP analysis was significantly lower than that of HCV ($P < 0.05$). The data indicate that: 1) GBV-C/HGV can be devoid of antigenic drift unlike HCV; 2) GBV-C/HGV has no HVR as seen in HCV in the presumed antigenic region; and 3) the sensitivity to IFN differs among GBV-C/HGV strains in the same hosts, as with HCV. ***J. Med. Virol. 55:109–117, 1998.***

KEY WORDS: GBV-C; HGV; HCV; SSCP; hypervariable region; interferon-α

INTRODUCTION

The newly discovered GB virus C/hepatitis G virus (GBV-C/HGV) is believed to be a member of the Flaviviridae, which includes hepatitis C virus (HCV) [Simons et al., 1995; Leary et al., 1996; Linnen et al., 1996]. GBV-C/HGV and HCV are thus related and share a common ancestor according to phylogenetic analysis, and have similar genomic structures [Leary et al., 1996; Ohba et al., 1996]. HCV has a hypervariable region (HVR) in the N-terminus of the envelope 2 (E2) region, and exists as a mixture of heterogeneous viruses so that it is termed a quasispecies in vivo [Oshima et al., 1991; Weiner et al., 1991]. The HVR is thought to be the neutralizing epitope, and the selection of escape variants from the HVR quasispecies pool facilitates the persistence of infection [Kato N et al., 1992; Kato N et al., 1994]. GBV-C/HGV infection can also persist for a long time [Masuko et al., 1996; Alter et al., 1997]. The E2 region of GBV-C/HGV is highly conserved, whereas contains replaceable amino acids in the N-terminus of the E2 region. This portion has been suggested to be the antigenic region by Takahashi et al.[1997]. Previous reports have indicated that GBV-C/HGV also exists as quasispecies with a lower degree of variation than HCV in the NS3 or NS5 regions [Pickering et al., 1997; Viazov et al., 1997], and has no HVR depends on the study of the comparison between the

The nucleotide sequences in this paper will appear in the DDBJ/EMBL/GenBank with the following accession numbers, AB005667 to AB005685 (GBV-C/HGV) and AB005686 to AB005716 (HCV).

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two strains isolated from a hemodialysis patient at an interval of 8.4 years [Nakao et al., 1997]. However, the existence of a mixture of heterogeneous viruses in the presumed antigenic region and the fluctuation of their population have not been confirmed. We investigated the heterogeneity of the presumed antigenic region in the E2 region of GBV-C/HGV and in the HVR of HCV by single-strand conformation polymorphism (SSCP) analysis for the immuno-competent hosts, and assessed the fluctuation of their population during persistent infection or those affected by interferon therapy.

PATIENTS AND METHODS

Patients

Six patients with chronic hepatitis who were positive for both GBV-C/HGV RNA and HCV RNA were studied. Two patients were observed for 4 years without any treatment (patients 1 and 2), and four patients received interferon-α (IFN) at 6 million units (MU) per day for 2 weeks followed by 6 MU thrice weekly for 22 weeks (patients 3–6). All patients were positive for anti-HCV (EIA-2, Ortho, Raritan, NJ), negative for HBsAg (AUSRIA II, Abbott Laboratories, North Chicago, IL), and antibody to human immunodeficiency virus (Abbott Laboratories). Serum samples were obtained from the two patients followed for 4 years at five time points yearly (time points I–V), and from four patients who received IFN therapy at three time points, before the start of IFN therapy (time point I), at the end point of IFN therapy (time point II), and 6 months after the cessation of IFN therapy (time point III). All samples were stored at –40°C until use.

RNA Extraction and cDNA Synthesis

Serum RNA was extracted from 100 μL using the SepaGene-RVR kit (Sanko, Tokyo), precipitated with isopropanol, and washed with ethanol. Complementary DNA (cDNA) was synthesized from the RNA samples at 37°C for 1 hour using Moloney murine leukemia virus reverse transcriptase (MMLV-RT; GIBCO BRL, Gaithersburg, MD) with random primers.

Detection of GBV-C/HGV and HCV

GBV-C/HGV RNA was detected by the reverse transcription polymerase chain reaction (RT-PCR) with primers derived from the 5′-untranslated region (UTR) [Kato T et al., 1997]. HCV RNA was also detected by RT-PCR with the primers derived from 5′-UTR [Okamoto et al., 1990].

Genotyping and Quantification of GBV-C/HGV and HCV

The genotypes of GBV-C/HGV were divided into three groups in this study, the GB type, the HG type and the Asian type, corresponding to genotypes 1, 2, and 3, respectively, of Muerhoff et al. [1996], using restriction fragment length polymorphism (RFLP) analysis [Mukaide et al., 1997]. The HCV genotype was determined by the mixed primer PCR method [Ohno et al., 1997]. Quantification of GBV-C/HGV was performed by competitive RT-PCR [Orito et al., 1997], and quantification of HCV by an Amplicor Monitor HCV test (Roche Diagnostic Systems, Branchburg, NJ).

PCR for SSCP Analysis

GBV-C/HGV RNA was detected by semi-nested PCR with specific primers based on the sequence of the E2 region. This region contains the presumed antigenic region, including two Asn-linked glycosylation sites and two pairs of Cys residues to form a loop structure exposed on the virion surface, as suggested by Takahashi et al. [1997]. The first round of PCR was performed with primers gE2f3 (5′-GCGCAACGGATTGTCATGGTCTTCC-3′) and gE2r3 (5′-CGATGATCCAAGTGCGGCTATGGTGCA-3′) for 30 cycles, consisting of denaturation for 1 minute at 94°C, annealing for 45 seconds at 60°C, and extension for 1 minute at 72°C. The second round of PCR was performed with primers gE2f3 and gE2r1 (5′-GGCCACTGATTTTGCCCGTGGCTCCA-3′) for 35 cycles using the same conditions as in the first round of PCR. The amplicons were separated by electrophoresis on 3% agarose gels, stained with ethidium bromide, and observed under ultraviolet light. The expected amplicon size was 293 bp (corresponding to nucleotides 1107 to 1399 of HGV PNF2161) [Linnen et al., 1996], containing the sequence coding for the presumed antigenic region (1221 to 1304 of HGV PNF2161) in the center.

HCV RNA was detected by semi-nested PCR with specific primers based on the sequence of the HVR. The first round of PCR was undertaken with primers cMS1 (5′- CACC GCATGGCWTGGGATAT-3′; W = A+T) and cMR2 (5′-CTGTTGATGTGCCAGCTGCC-3′) for 35 cycles, consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 50°C, and extension for 2 minutes at 72°C. The second round of PCR was performed with primers cMS2 (5′-TTGGGATATGATGATGAACTGG-3′) and cMR2 for 25 cycles, consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C, and extension for 1 minute at 72°C. The expected amplicon size was 324 bp (corresponding to nucleotides 1293 to 1616 of HCV-J) [Kato N et al., 1990], containing the sequence coding for the HVR (1469 to 1549 of HCV-J) in the latter half.

Single-stranded cDNAs of GBV-C/HGV and HCV were amplified by asymmetric PCR with the first PCR product as a template. Asymmetric PCR was performed with the same primers, the same concentration for sense primer and a tenth dilution of the anti-sense primer, and the same conditions as for the second round of PCR.

SSCP Analysis

Five μl of single-stranded cDNA was mixed with 10 μl of loading buffer containing 95% formamide, 20 mM EDTA, and 0.1% xylene cyanol. The mixture was loaded onto non-denaturing polyacrylamide gel (MDE gel, FMC Bioproducts, Rockland, ME) and electrophoresis was carried out at 20°C, 300 V for 3 hours. The gel

TABLE I. Backgrounds of Patients Infected With GBV-C/HGV and HCV

Patient	Age (yr)	Sex	Virus	Genotype	Viral RNA level I	II	III	IV	V	
1	77	M	GBV-C/HGV	Asian	5.5	7.0	3.5	4.0	4.0	(log copies/ml)
			HCV	2a	63	170	37	83	110	(K copies/ml)
2	59	M	GBV-C/HGV	Asian	5.0	4.5	6.5	6.5	6.5	(log copies/ml)
			HCV	1b	450	1000	680	850	440	(K copies/ml)
3	54	F	GBV-C/HGV	Asian	7.0	5.0	7.0			(log copies/ml)
			HCV	1b	300	190	400			(K copies/ml)
4	28	M	GBV-C/HGV	Asian	7.5	4.0	7.5			(log copies/ml)
			HCV	2a + 2b	120	ND[a]	450			(K copies/ml)
5	40	M	GBV-C/HGV	Asian	7.0	ND	8.0			(log copies/ml)
			HCV	1b	590	ND	<1.0			(K copies/ml)
6	27	F	GBV-C/HGV	Asian	7.0	ND	7.0			(log copies/ml)
			HCV	1b	650	ND	850			(K copies/ml)

[a]ND, not detected.

was stained with ethidium bromide and bands were detected under ultraviolet light. Separated bands were excised from the gel, and cDNA was recovered by incubation at 60°C overnight. Recovered cDNA was amplified by PCR using the same primers and conditions as for the second round of PCR.

Calculation of Genetic Distance

The nucleotide sequences of the amplicons were determined by the dideoxy chain termination method. In order to quantify the degree of variation among GBV-C/HGV and HCV strains in each patient, genetic distances, and the number of nucleotide substitutions per site at each nucleotide position, were calculated between all possible pairs of strains by the six-parameter method using the molecular evolutionary software system ODEN, version 1.1.1. [Gojobori et al., 1982; Ina, 1994].

Statistical Analysis

For statistical analysis, the Mann-Whitney's U test was used.

RESULTS

SSCP Analysis for Patients Without any Treatment

The backgrounds of the patients, their viral genotypes and their viral RNA levels are shown in Table I. Two patients (patients 1 and 2) were infected with the same Asian type of GBV-C/HGV and their viral titers were similar. They were infected, however, with different genotypes of HCV, this being 2a in patient 1 but 1b in patient 2, and the HCV viral titer was higher in the latter. The ALT levels of patient 1 were higher than those of patient 2, and fluctuated during the follow-up period.

The results of SSCP analysis are shown in Figure 1a. Single-stranded cDNA for GBV-C/HGV and HCV were separated into 1–3 bands on gels at each time point during the follow-up period of 4 years. Although the nucleotide sequences of GBV-C/HGV represented by each band exhibited one to four differences, the deduced amino acid sequences demonstrated no or only one difference. Moreover, in the presumed antigenic region, there was no variation in the amino acid sequence (Figs. 2 and 3a). The predominant strain of GBV-C/HGV in these patients did not change during the follow-up period (patient 1B, patient 2A).

The respective nucleotide and amino acid sequences of HCV represented by each band exhibited differences, and the predominant strain varied during the follow-up period (Fig. 3b). The HCV genotype appeared to be independent with the population and fluctuation of the GBV-C/HGV strains. The number of distinct bands on gels tended to be related to the viral titer of HCV, but this was not the case for GBV-C/HGV.

SSCP Analysis of Patients With IFN Treatment

The four tested patients (3–6), were also infected with the Asian type of GBV-C/HGV and their viral titers before IFN therapy were similar. One (patient 4) was infected with HCV genotypes 2a+2b, and the others with 1b. The viral titers before IFN administration were almost the same (Table I). The ALT levels were increased before IFN therapy, in particular, those in patient 3, and decreased at the end point of IFN administration. Except in one case (patient 3), the ALT levels had again increased 6 months after the cessation of IFN therapy. Virologically, GBV-C/HGV RNA was undetectable at the end point of IFN therapy in to patients (5 and 6), again becoming detectable 6 months thereafter. HCV RNA was also undetectable at the end point of IFN therapy in three patients (4–6), and became detectable 6 months after therapy cessation (Table I).

As a result of SSCP analysis, GBV-C/HGV cDNA was separated into 1–5 bands on gels (Fig. 1b). The cDNAs represented by these bands had 1–4 different nucleotide sequences and the deduced amino acid sequences had no or only one difference . The amino acid sequences in the presumed antigenic region exhibited no differences, as with the patients not undergoing IFN treatment (patients 1 and 2). Most HCV strains, distinct to 2–4 bands on gels, had different amino acid sequences in the HVR for the same patient. Two

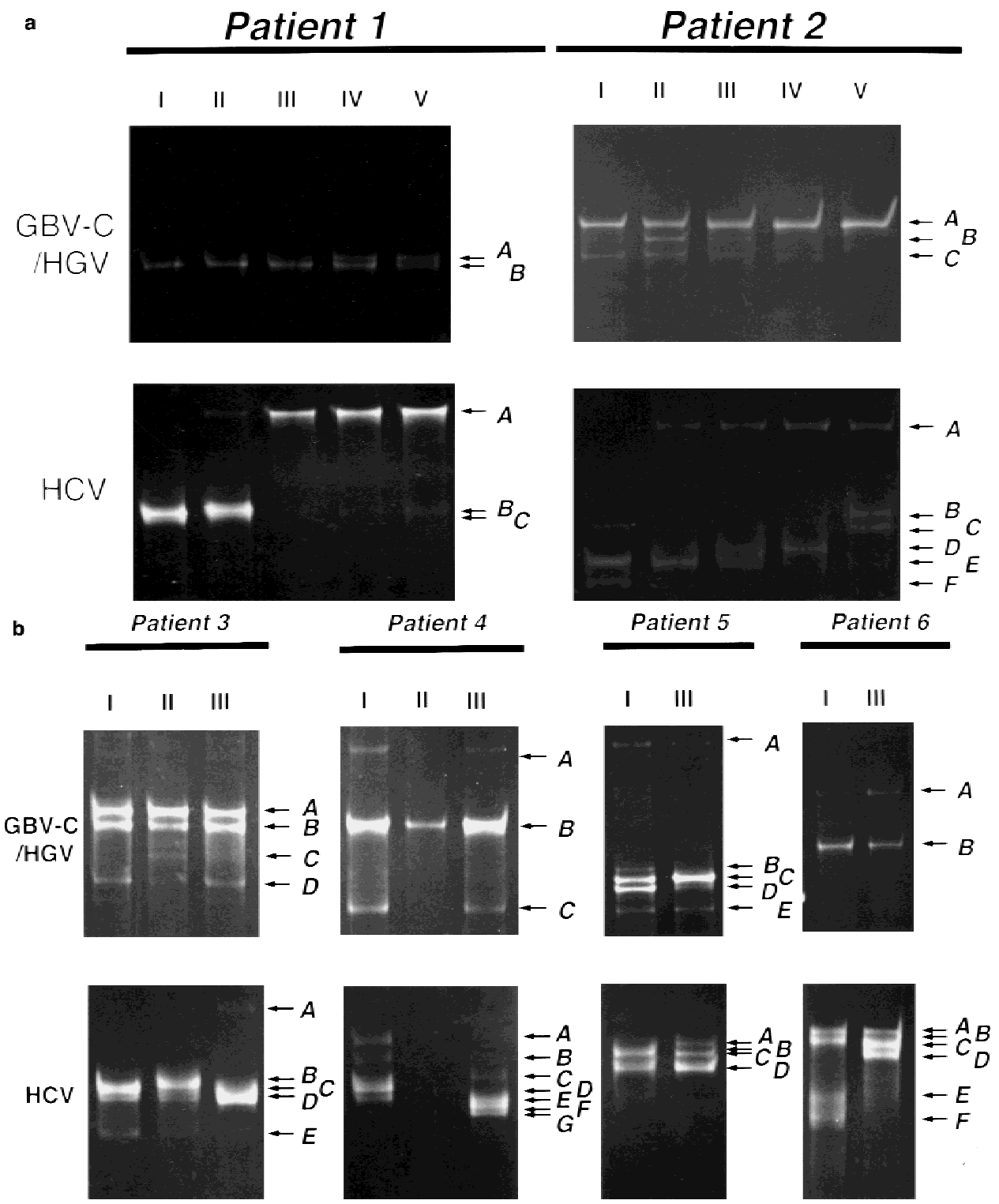

Fig. 1. SSCP analysis of patients with GBV-C/HGV and HCV. The numbers I to V indicate the yearly time points at which serum samples were obtained from patients 1 and 2 followed for 4 years, and the time points I to III for patients 3 to 6 are before the start of IFN therapy (time point I), at the end point of IFN therapy (time point II), and 6 months after the cessation of IFN therapy (time point III).

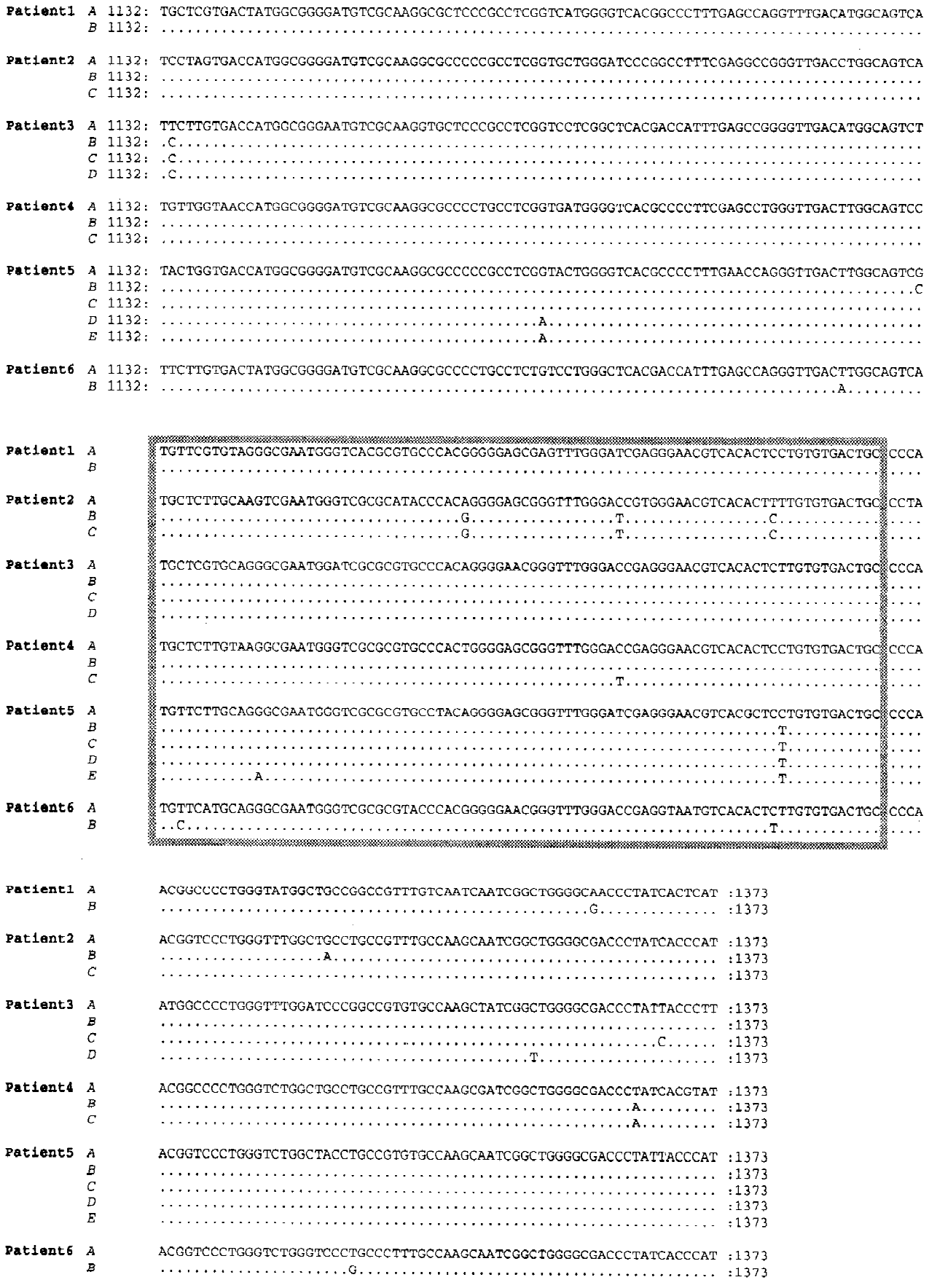

Fig. 2. Alignment of nucleotide sequences of GBV-C/HGV in the E2 region. These sequences were obtained from bands separated by SSCP for patients 1–6. The box indicate the presumed antigenic region. Identical nucleotides are indicated by dots. The nucleotide sequences are numbered according to the sequence for HGV PNF2161 (accession number U44402).

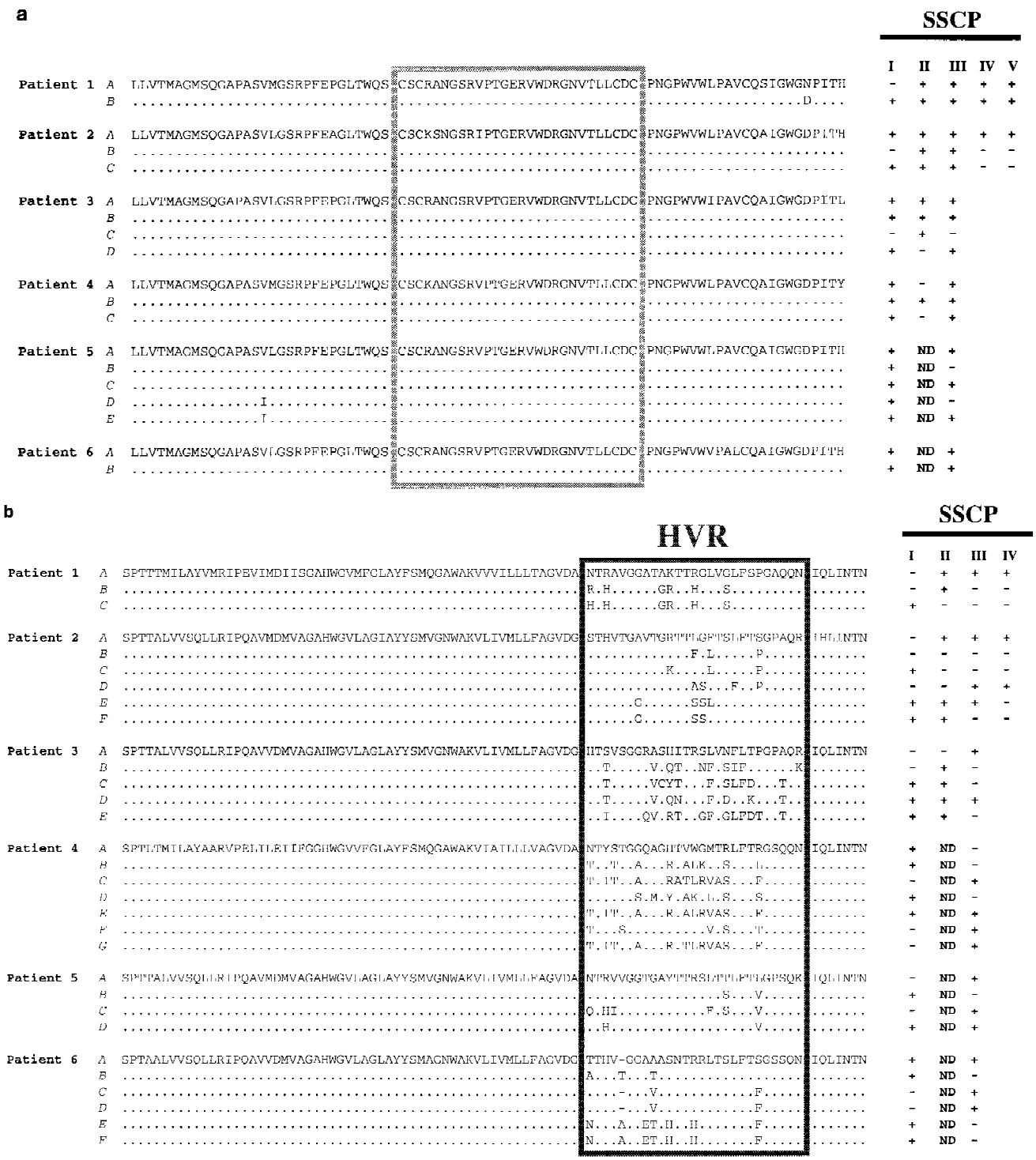

Fig. 3. Alignment of deduced amino acid sequences of GBV-C/HGV and HCV (**a** and **b,** respectively). Each sequence was deduced from the nucleotide sequences obtained from the SSCP bands shown in Figure 1. The boxes indicate the presumed antigenic region of GBV-C/HGV and the HVR of HCV. Identical nucleotides and gaps are indicated as dots and dashes, respectively. On the right side of the sequences, results of SSCP at each time point are shown (+, positive, –, negative, ND, not detected).

strains of GBV-C/HGV (patient 5B and D) disappeared at the end point of IFN therapy and were also not detected 6 months after the IFN therapy, whereas others persisted. One strain of GBV-C/HGV (patient 1C) appeared at the end point of IFN therapy. There was no characteristic difference in the nucleotide or amino acid sequences of the presumed antigenic region between the disappeared and persisting strains (Fig. 3a). The HCV strains population changed in the period before and after IFN therapy (Fig. 3b).

TABLE II. Genetic Distances Among Strains of GBV-C/HGV and HCV

Patient		GBV-C/HGV	HCV	Partial region of HCV	
				HVR	Not containing HVR
1	Genetic distance[a]	0.0043	0.0386	0.1138	0.0185
	No. of strains	2	3	3	2
2	Genetic distance[a]	0.0113	0.0174	0.0634	0
	No. of strains	3	6	6	1
3	Genetic distance[a]	0.0059	0.0775	0.2229	0.0265
	No. of strains	4	5	5	5
4	Genetic distance[a]	0.0054	0.0520	0.1693	0.0103
	No. of strains	3	7	7	3
5	Genetic distance[a]	0.0077	0.0197	0.0505	0
	No. of strains	5	4	4	1
6	Genetic distance[a]	0.0159	0.0301	0.0945	0.0117
	No. of strains	2	6	6	3
Mean genetic distance		0.0084 ± 0.0040	0.0392 ± 0.0207	0.1191 ± 0.0602	0.0110 ± 0.0094
			*		
				*	
					**

*$P < 0.05$.
**P = not significant.
[a]Genetic distances are indicated as averages between all possible pairs of strains in each patient.

Genetic Distance Among Strains

The average genetic distances between all possible pairs of strains in each patient are shown in Table II. The genetic distances among GBV-C/HGV strains were lower than those for HCV in each patient, and the mean genetic distance of GBV-C/HGV was significantly lower than that of HCV ($P < 0.05$). Evaluation between GBV-C/HGV and a partial region of HCV, containing and not containing the HVR, revealed that the mean genetic distance of GBV-C/HGV was significantly lower than that of the region containing the HVR ($P < 0.05$), but similar to that of the region not containing the HVR.

DISCUSSION

SSCP analysis is a method to detect nucleotide differences in single-stranded cDNA as mobility shifts depending on their sequence-specific three-dimensional conformations, and can detect a single point mutation [Orita et al., 1989]. This method has advantages in that an exact evaluation of HCV quasispecies populations in vivo can be carried out compared with the cloning of a few samples, and it was usefulness to form a basis in identification for the interferon sensitivity determining region (ISDR) of HCV [Enomoto et al., 1994, 1996]. The hydropathy profile for the envelope protein of HGV PNF2161 (accession number U44402) displays some hydrophilic peaks (data not shown), and one hydrophilic portion, located at the N-terminus of E2 region as like as HVR of HCV, contains two Asn-linked glycosylation sites, and two pairs of Cys residues to form a loop structure exposed on the virion surface. Therefore, this region has been regarded as a antigenic region [Takahashi et al., 1997]. Thus, the evaluation to this presumed antigenic region by SSCP analysis revealed some important facts about the heterogeneity of GBV-C/HGV and its modes of existence in vivo.

Unlike HCV, GBV-C/HGV can be devoid of antigenic drift during long-term infection. The predominant strain of GBV-C/HGV did not change during the follow-up period of 4 years in two patients, although the predominant strain of HCV did vary in these two patients. Moreover, the GBV-C/HGV strains in the same host exhibited no amino acid differences and no HVR, as seen in HCV, in the presumed antigenic region. The HVR of HCV is known to contain the neutralizing epitope and changes in this region enable the virus to escape detection by a host's immune system. Although GBV-C/HGV, like other RNA viruses, exists as a mixture of heterogeneous viruses in vivo, the deduced amino acid sequences of these viruses did not change in the presumed antigenic region. The mean genetic distance among GBV-C/HGV strains was found to be significantly lower than that of HCV, and similar to that of HCV not containing the HVR. A previous report showed a lower sequential variability in the coding region and 3′-UTR [Erker et al. 1996; Viazov et al., 1997]. Therefore, our findings are consistent with these data and confirm it also in the presumed antigenic region.

GBV-C/HGV can be devoid of antigenic drift and its lack of the HVR, suggests that its method of maintaining persistent infection is different from that of HCV. It has been reported that GBV-C/HGV forms an immune complex with antibodies less frequently than does HCV, and GBV-C/HGV in circulation is covered by lipoproteins which can prevent it from being eliminated by the host's immune response [Hijikata and Mishiro, 1996; Sato et al., 1996]. These may be associated with another mechanism which may be used to maintain persistent infection. On the other hand, the detection of antibody to GBV-C/HGV E2 protein, the marker for past infection, revealed that some cases of recovery

from GBV-C/HGV infection can be found among low risk patients who were negative for GBV-C/HGV RNA [Pilot-Matias et al., 1996; Tacke et al., 1997]. The absence of the HVR possibly renders GBV-C/HGV prone to elimination more easily by the immune system than HCV.

Finally, the sensitivity to IFN is different among GBV-C/HGV strains in the same host, in the same manner as for HCV [Mizokami et al., 1994]. Some strains of GBV-C/HGV disappeared as a result of IFN therapy, whereas other strains exhibited resistance to IFN treatment in the same hosts. However, the amino acid sequences in the E2 region among these strains were identical, and there were no characteristic differences in nucleotide sequences between IFN sensitive and non-sensitive strains. Regions in which there were differences, such as the ISDR of HCV in the NS5A, may be related to the IFN sensitivity of GBV-C/HGV [Enomoto et al., 1996]. Identification of the ISDR of GBV-C/HGV and comparison with that of HCV should shed further light on its structure and function of the ISDR. The investigation for the mechanism of persistent infection or the IFN sensitivity of GBV-C/HGV and comparison with HCV will help to clarify the characteristics of HCV, which exhibits liver pathogenicity and is related to GBV-C/HGV. Further studies are required to elucidate the mechanism underlying the persistent infection and resistance to IFN therapy of GBV-C/ HGV and HCV.

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